Plasmid Profile and Protease Activity of *β*-Lactams Resistant Thermotolerant Soil Isolate *B. cereus* BC2 from the *Bacillus cereus* Group Species

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Abstract: The aim of the current study was to isolate and investigate one of the most common genera of Gram positive spore forming bacteria, genus *Bacillus*. *Bacillus cereus* BC2 was grown well on nutrient agar plates (pH 8) after 24 hrs of incubation at 55°C. This isolate was characterized by producing extracellular protease which showed activity at 55°C both *in vivo* & *in vitro*. The optimum enzyme production was at pH 8, incubation temperature 55°C, and NaCl concentration of 5% (w/v) where the highest protein concentration and the highest enzyme activity were recorded. The activity of the partially purified enzyme reached to 160.00 UmL^{-1} with about 3.10 mg mL^{-1} protein concentration recorded. The isolate *Bacillus cereus* BC2 was resistant to the *β*-Lactam antibiotics tested including ampicillin. Finally, plasmid DNA extraction from the isolate under study and visualization through agarose gel electrophoresis showed the absence of any free plasmid molecules within the vegetative cells of this isolate.
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1. Introduction

Most species of the genus *Bacillus*, facultative anaerobe, endospore-forming, rod–shaped, Gram-positive bacteria, are saprophytic and are widely distributed in nature, particularly in soil. *Bacillus anthracis, B. cereus, B. thuringiensis, B. mycoides* and more recently *B. pseudomycoides* and *B. weihenstephanensis* comprise the *B. cereus* group (Manzano *et al*., 2003). The most common and well studied of these are *B. anthracis, B. cereus,* and *B. thuringiensis*. Although they have similar characteristics, they are distinguishable as *B. cereus* is most motile, *B. thuringiensis* produces crystal toxins, and *B. anthracis* is nonhemolytic. Phylogenetic analysis shows that the *B. cereus* group of bacteria is closely related group (Stenfors *et al*., 2008).

The *Bacillus cereus* group is a very homogeneous cluster, reported to have 99% similarity in their 16s rRNA sequences (Ash *et al*., 1991), a level of homology usually interpreted by bacterial taxonomists as being indicative of a single species. A practical consequence of this is that they are very difficult to differentiate, even using molecular biology techniques. Helgason *et al*., (2000) show by multilocus enzyme electrophoresis and by sequence analysis of nine chromosomal genes that *B. anthracis* should be considered a lineage of *B. cereus*. This determination is not only a formal matter of taxonomy but may also have consequences with respect to virulence and the potential of horizontal gene transfer within the *B. cereus* group. Major advances in the filed of differentiating these group of organisms were developed by Lasch *et al*., (2009).

The hallmark characteristic of the genus *Bacillus* and related genera is the ability to form environmentally resistant spores. The spore is the survival form of the *Bacillus* and spores of some *Bacillus* isolates are extremely resistant to heat, chemicals, radiation and desiccation (Setlow, 2006). *B. cereus* is mesophilic, growing optimally at temperatures between 20°C and 40°C, and is capable of adapting to a wide range of environmental conditions (Vilain *et al*., 2006).

Bacillus cereus is present in almost every environment including vegetable production areas (Murray *et al*., 2002). The importance of the bacterium arises because it can cause foodborne disease (Jenson and Moir, 2003). As an ever-present bacterium, small amounts are consumed by humans from foods. Therefore, it is also contributor to the human intestinal microflora (Jensen *et al*., 2003). In addition *B. cereus* is widely known to affect humans by causing food poisoning and infections as an opportunistic pathogen.

Extracellular enzyme synthesis in the genus *Bacillus* gained interest many decades ago, see (Priest, 1977) for reviews. Among the important protease producing bacteria are species of *Bacillus*. Among all bacterial species, bacilli play an important role in production of alkaline protease owing to their chemoorganotrophic nature (Miyaji *et al*., 2006). The enhancement of protease production by genetic manipulation has been well studied in *B. cereus*, *B. subtilis*, *B. stearothermophilus*, etc. by a number of

researchers, which additionally highlights the importance of this enzyme (Rao *et al*., 1998).

Proteases are the most important industrial enzymes that execute a wide variety of functions and have various important biotechnological applications (Mohen *et al*., 2005). Many of the organisms produce more than one kind of protease. The type of proteolytic enzyme formed may depend on the composition of the medium. Culture conditions play significant role on growth and production of protease by bacteria. The genus *Bacillus* contains a number of industrially important species and approximately half of the present commercial production of bulk enzymes derives from the strains of *Bacillus* sp. (Beg and Gupta, 2003). These strains are specific producers of extracellular proteases (Singh *et al*., 2001) and can be cultivated under extreme temperature and pH conditions to give rise to products that are, in turn, stable in a wide range of harsh environments (Han and Damodaran, 1997).

Identification and classification of plasmids are especially important in medicine, because genes for clinically important traits, such as drug resistance and virulence factors, are frequently present in plasmids (Couturier *et al*., 1988). *B. cereus* has a diverse range of plasmids that vary in size from 5 to 500 kb and is known to have more than one plasmid with only a few that is associated with pathogenesis. *B. cereus* ATCC has a pXO1 plasmid that is found in *B. anthracis* (Rasko *et al*., 2005). The genes for the toxins in *B. cereus,* which cause human illness, are plasmid-borne meaning they are transferable between cells (EFSA, 2004). Conjugative behavior shows that these *Bacillus* species are closely related and plasmid transfer within the *B. cereus* group has been demonstrated in different environments (Van der Auwera *et al*., 2007).

More frequently resistance is due to the presence of additional gene(s) as extrachromosomal DNA known as R-factors (plasmids) (Choudhury, 1995). A large variety of specific biochemical functions, such as fertility, resistance to antimicrobial drugs, production of bacteriocins, and production of toxins, have been attributed to some plasmids (Bernhard *et al.*,1978).

A number of plasmids have been isolated as covalently closed circular DNAs from strains of *Bacillus cereus* and *B. subtilis* by Bernhard *et al.*, (1978). Most of the *B. cereus* strains contained two or more plasmids. Two streptomycin-resistant strains of *B. subtilis* harbored plasmids which were, however, not correlated with the antibiotic resistance. The plasmid carrying resistance to tetracycline, pBC16, which was originally isolated from *B. cereus*, could be subsequently transformed in *B. subtilis*, where it is stably maintained.

Extrachromosomal properties must therefore occur frequently in *B. cereus*. It appears unlikely that the common physiological properties of *B. cereus*, such as resistance to penicillin, colistin, and polymyxin and hemolytic activity, are plasmid inherited, since the few isolates of *B. cereus* that do not harbor plasmids possess these properties as well. There are, however, additional properties in some of *B. cereus* strains analyzed that are likely to be extrachromosomally inherited. In particular, Tetracycline resistance, which could be cured and lost by ethidium bromide, accompanied by the loss of a small plasmid carried by *B. cereus* strain (Bernhard *et al.*,1978). Recently, *β*-lactamase type I occurs in the sporulated form in penicillin-resistant *B. cereus* was observed (Fenselau *et al*., 2008). A common cause of antibiotic resistance in bacteria is an increased abundance of β-Lactamase genes that are found in the wild-type genomes of many bacteria, including *Bacillus* species (Majiduddin *et al*., 2002).

The aim of the current study was to isolate and investigate one of the most common genera of Gram positive spore forming bacteria, genus *Bacillus*, specially the *Bacillus cereus* group species, due to its very high economic importance. High temperature tolerance, proteolytic activity, antibiotic resistance, and plasmid detection all were carried out to characterize the obtained isolate.

2. Material and Methods

2.1. Isolation of aerobic spore formers from soil samples

The soil samples were collected aseptically from the upper most 0-5 cm soil layer of different vegetable cultivated localities in Giza governorate, Egypt. About 1.0 g of soil sample was transferred to 99.0 ml sterilized normal saline in 250 ml conical flask and agitated (100 rpm) at 37°C for 15 minutes on water bath shaker (Eyela, Japan). The sample was then heated at 70°C for 15 minutes to destroy all the vegetative microbial cells. The soil suspension was then diluted in serial up to 10^{-7} dilutions. One ml of each dilution was poured into Petri plates containing nutrient agar (Oxoid) medium of pH 8.0. The inoculated plates were then incubated at 55°C for 48 hours.

2.2. Characterization and identification of the isolate

All characterization and identification tests as well as the used media were carried out according to Sneath, (1986) and according to the methods mentioned by Priest *et al*., (1988). Also, the identification was assessed by using API stripes system according to Logan and Berkeley, (1984). The used system was API 50 CHB/20E to determine the

phenotypic characteristics of the isolate. This combination of API 50 CHB with API 20 E systems, (bioMerieux, France), were used following the manufacturers instructions to study the sugar fermentation and the biochemical characteristics of the isolate. The results were analyzed using the API Web database (https://apiweb.biomerieux.com) for species level identification. In addition, endospore and crystal staining were carried out according to Chilcott and Wigley, (1988). Sporulated cultures on nutrient agar were used for the structural staining. Air-dried smear was heat fixed and incubated at 100°C for ten minutes. The hot slide was placed into amido black 10B (1.5 g in 100 ml 35% glacial acetic acid) solution for ten minutes. Excess stain was washed thoroughly in tap water, air-dried; flood with giemsa stain for 2-5 min. Excess stain was washed thoroughly in tap water, air-dried and observed under oil immersion objective with the aid of a phase contrast microscope. Crystals stain dark blue and spores pale to light blue with dark margin. Also, the haemolysis pattern was detected on TSA plates containing sheep blood (TSB 30 g/L, Bacto Agar 15 g/L, defibrinated sheep blood 50 ml/L). The plates were inoculated with the aerobic spore former isolate and incubated for 24 h at 37°C. The plates were then evaluated and scored for level of haemolysis.

2.3. Protease activity of the isolate (qualitative assay)

Protease activity of the isolate was measured by caseinolytic zone diameter (mm) (Ellaiah *et al.*, 2002) in glucose yeast extract (GYE) broth (pH 8) containing (gl^{-1} distilled water): glucose, 15.0; yeast extract, 5.0 and $CaCl_2.2H_2O$, 0.2. One hundred μl of 24 h at 55°C culture broth of the isolate was loaded in the agar wells created on skim milk agar plates (pH 8) and incubated for 5 h at 55°C for *in vitro* assay. Also, *in vivo* assay was carried out on skim milk agar plates containing skim milk 1.0 %, peptone 0.1 %, sodium chloride 0.5 %, and agar 2.0 %. The pH of the medium was adjusted at 8.00 with 1 N HCl/ 1N NaOH before sterilization at 121°C for 15 minute. The inoculated plates were then incubated at 55°C for 48 hrs and observed for zones of clearance which indicate proteolytic activities.

2.4. Protease activity quantitative assay and protein determination

Extracellular protease activity was determined by a modified method of Yang and Huang, (1994). The reaction mixture containing 1 ml of 1.0 % casein solution in 0.05 M Glycine-NaOH buffer having pH 8 and 1 ml of a given enzyme solution (after growth on GYE broth) were incubated at 55°C for 20 minutes and the reaction was then stopped with 3 ml of 10 % tri-chloroacetic acid (TCA). The mixture was centrifuged at 15,000 rpm (4°C) for 10 min, and the supernatant was used to estimate the amount of free tyrosine as per Lowry *et al.*, (1951) using tyrosine as standard. The absorbance of the liberated tyrosine in the supernatant was measured at 280 nm by UV/VIS spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that liberates 1.0 µg of tyrosine min^{-1} ml^{-1} under the assay conditions. Also, the total protein concentration of the supernatant sample was determined using the same procedure of Lowry *et al.*, (1951) with bovine serum albumin as a standard.

2.5. Production of enzyme, partial purification, and effect of environmental factors

Fifty ml of nutrient broth (Oxoid), GYE broth, and TS broth (TSB) having pH 8 was inoculated with the isolate in triplicates and incubated at 55°C for 48 hrs at 140 rpm. The inoculated broth was then centrifuged at 10000 rpm for 10 minutes at 4.0°C. The supernatant was used to determine the protease activity. The partial purification of enzyme has been then carried out by precipitating the cell free supernatant with different concentrations of ammonium sulphate (from 10 - 80%). The precipitate was dissolved in small amount of 25 Mm glycine-NaOH buffer (pH-8) and dialyzed over night against the same buffer. In addition, the effect of environmental variables on the growth and enzyme production and activity were tested. The tested variables included different temperatures, pH values, and NaCl concentrations. The isolate was grown in TSB at 30°, 35°, 40°, 45°, 50°, 55° and 60°C to determine its optimum growth temperature and enzyme activity. The isolate was also grown at various pH levels from 6 to 11. The isolate was finally grown in a TSB solution with 1%, 3%, 5% and 7% (w/v) Sodium Chloride. The activity of the protease was then measured as per assay procedure.

2.6. Antibiotic resistance and sensitivity test

The antibiotic resistance test of the isolate was studied by disc diffusion method (Bauer *et al.*, 1966). Prior to the resistance test, the isolate was stimulated on blood agar plates. Then four to five colonies were resuspended in 2 ml of Mueller-Hinton broth (Oxoid) and further diluted as recommended by NCCLS (1999) with the same medium under spectrophotometric control to obtain suspension with density of approximately 5×10^6 CFU/ml. The antibiotics (µg/disc) used were cephalexin (30), cefaclore (30), penicillin (10), ampicillin (10), amoxycillin (10), erythromycin (15), lincomycin (15), amikacin (30), chloramphenicol (30),

kanamycin (30), neomycin (30), tetracycline (30), and streptomycin (10). Antibiotic discs were placed over freshly prepared bacterial lawn on Mueller Hinton agar plates inoculated with the tested isolate (grown for 16 h at 35°C) and the plates were kept at 4°C for 3 h for antibiotic diffusion. The plates were then incubated at 35°C for 16 h and the growth of the bacteria was observed. The presence of a clear zone around the disc was the index of sensitivity to the antibiotic. The test results of antibiotic sensitivity were determined according to the inhibition zone diameter (Çetin and Gurler, 1989). The isolate was classified as sensitive or resistant by the presence/absence of inhibition zone of growth around the antibiotic discs.

2.7. Plasmid isolation and detection test

Plasmid DNA was extracted from the isolate under study according to the methods described by Sambrook *et al*., (1989). The cells were grown aerobically overnight in Luria broth (LB) with vigorous shaking (200 rpm) at 35°C with addition of ampicillin (10 µg/ml). The cells were then collected by centrifugation and washed twice before extraction. Plasmid DNA was then isolated by using the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocols. Plasmid DNA was separated by gel electrophoresis in 0.8% agarose with 10 Kbp DNA Ladder (Life Technologies, United Kingdom) as a molecular weight marker. The gel was analyzed by using the GeneGenius gel documentation and analysis system (SynGene Laboratories, Cambridge, United Kingdom).

3. Results

3.1. Isolation of aerobic spore formers from soil samples

From the previously mentioned collected soil samples, a number of different isolates were obtained, and, from a combination of these samples, only one isolate was selected to carry out the current investigation. The selected isolate was belonged to the Gram positive spore forming aerobic bacteria. This isolate was designated as the most thermotolerant isolate among all the obtained isolates and also sought to belong to the *Bacillus cereus* group species so it was named isolate BC2. So, this isolate was selected for identification and to investigate its protease activity, antibiotic resistance pattern, and the possible existing plasmid DNA molecules within the vegetative cells of this isolate. The initiative was to come closer to one from this very important and highly economic group of bacteria. The isolate BC2 was grown well on nutrient agar plates (pH 8) after 24 hrs of incubation at 55°C.

3.2. Characterization and identification of the isolate

As previously mentioned all the required characterization and identification tests were carried out according to Sneath, (1986) and according to the methods mentioned by Priest *et al*., (1988). Table (1) show the morphological, physiological, & biochemical characteristics of the isolate BC2. This isolate was characterized by Gram positive rod shaped cells with endospores, aerobic growth, and high motility. Central, Ellipsoidal endospores were present but no parasporal crystals were observed using the staining procedure and examination under a phase contrast microscope. Also, the haemolysis pattern was detected on TSA plates to be *β*-hemolytic. This isolate was characterized by producing a very powerful protease hydrolyzing gelatin and casein very efficiently. Finally, this isolate also characterized by producing Lecithinase and grow at the different NaCl concentrations, pH values, and the temperature degrees tested. The rest of the results were presented in table (1). Also, the identification was assessed by using API system comprised of API 50 CHB/20E to determine the phenotypic characteristics of the isolate. This combination of API 50 CHB with API 20 E systems, (bioMerieux, France), were used following the manufacturers instructions to study the sugar fermentation and the biochemical characteristics of the isolate. Tables 2 & 3 show the obtained results for fermentation tests and enzymatic reactions, of the isolate BC2, which were analyzed using the API Web database (https://apiweb.biomerieux.com) for species level identification. Since, identification at the species level is considered acceptable with a percentage of similarity greater than 75%. So, the isolate BC2 was identified as *Bacillus cereus* with a high percentage of similarity according to the profile pattern of the bioMérieux identification program and named *B. cereus* BC2.

3.3. Protease activity of the isolate BC2 (qualitative assay)

Extracellular protease activity of the isolate was measured qualitatively by caseinolytic zone diameter (mm) in (GYE) broth (pH 8). One hundred *µl* of 24 h old broth culture of the isolate BC2 at 55°C was loaded in the agar wells created on skim milk agar plates (pH 8) and incubated for 5 h at 55°C for *in vitro* assay and yielded a clear zone of hydrolysis reached to 20 mm in diameter. Also, *in vivo* assay was carried out on skim milk agar plates (pH 8) and incubated at 55°C for 24 hrs and yielded a clear zone of hydrolysis reached to 25 mm in diameter.

Table 1. Morphological, physiological, & biochemical characteristics of the isolate *B. cereus* BC2.

Character	Character state
Rod shaped	Positive
Endospores produced	Positive
Spore shape	Ellipsoidal
Spore position	Central
Parasporal crystals	Negative
Gram's stain	Positive
Catalase	Positive
Anaerobiosis	Facultative
Motility	Highly motile
Acid from D-glucose	Positive
Acid from D-xylose	Negative
Acid from D-mannitol	Negative
Gas from D-glucose	Negative
Hydrolysis of casein	Positive
Hydrolysis of gelatin	Positive
Hydrolysis of starch	Weakly positive
Utilization of citrate	Weakly positive
Degradation of tyrosine	Positive
Deamination of phenylalanine	Negative
Lecithinase Egg-yolk reaction	Positive
Nitrate reduction	Positive
Indole formation	Negative
Growth at pH 6.8	Positive
Growth at pH 5.7	Positive
Growth in NaCl 2% (w/v)	Positive
Growth in NaCl 5% (w/v)	Positive
Growth in NaCl 7% (w/v)	Positive
Growth in NaCl 10% (w/v)	Weakly positive
Growth at 5°C	Negative
Growth at 10°C	Negative
Growth at 30°C	Positive
Growth at 40°C	Positive
Growth at 50°C	Positive
Growth at 55°C	Positive
Growth at 60°C	Weakly positive
Growth at 65°C	Negative
Growth with Lysozyme present	Positive
Lysine decarboxylase	Negative
Lipase (olive oil)	Positive
Hydrolysis of Tween 80	Positive
Hydrolysis of urea	Weakly positive
Gas from Nitrate	Positive
Acid from glycerol	Negative
Acid from Inositol	Negative
Acid from Lactose	Negative
Acid from Maltose	Positive
Acid from Sucrose	Negative
Utilization of acetate	Weakly positive
Utilization of lactate	Negative
Utilization of tartarate	Negative

Table 2. Fermentation test results obtained from the API for the isolate *B. cereus* BC2.

No.	Test	Result
1	Glycerol	Negative
2	Erythritol	Negative
3	D-arabinose	Negative
4	L-arabinose	Negative
5	D-Ribose	Positive
6	D-Xylose	Negative
7	L-xylose	Negative
8	D-Adonitol	Negative
9	Methyl-βD-Xylopyranoside	Negative
10	D-Galactose	Negative
11	D-Glucose	Positive
12	D-Fructose	Positive
13	D-Mannose	Negative
14	L-Sorbose	Negative
15	L-Rhamnose	Negative
16	Dulcitol	Negative
17	Inositol	Negative
18	D-Mannitol	Negative
19	D-Sorbitol	Negative
20	Methyl-αD-Mannopyranoside	Negative
21	Methyl-αD-Glucopyranoside	Negative
22	N-Acetyl-Glucosamine	Positive
23	Amygdalin	Negative
24	Arbutin	Negative
25	Esculin ferric citrate	Negative
26	Salicin	Negative
27	D-Cellobiose	Negative
28	D-Maltose	Positive
29	D-Lactose	Negative
30	D-Melibiose	Negative
31	D-Sucrose	Negative
32	D-Trehalose	Positive
33	Inulin	Negative
34	D-Melezitose	Negative
35	D-Raffinose	Negative
36	Starch	Negative
37	Glycogen	Negative
38	Xylitol	Negative
39	Gentiobiose	Negative
40	D-Turanose	Negative
41	D-Lyxose	Negative
42	D-Tagatose	Negative
43	D-Fucose	Negative
44	L-Fucose	Negative
45	D-Arabitol	Negative
46	L-Arabitol	Negative
47	Potassium Gluconate	Positive
48	Potassium 2-KetoGluconate	Negative
49	Potassium 5-KetoGluconate	Negative

Table 3. Enzymatic reaction results obtained from the API for the isolate *B. cereus* BC2.

No.	Test	Result
1	Beta-galactosidase	Negative
2	L-arginine for Arginine Dihydrolase	Positive
3	L-lysine for Lysine Decarboxylase test	Negative
4	L-ornithine for Ornithine Decarboxylase	Negative
5	Trisodium citrate for Citrate utilization	Negative
6	Sodium thiosulfate for H_2S production	Negative
7	Urea for Urease	Negative
8	L-Tryptophane for Tryptophane deaminase	Negative
9	L-tryptophane for Indole production	Negative
10	Sodium pyruvate for acetoin production	Negative
11	Gelatin for Gelatinase enzyme	Positive
12	Nitrate reduction (N_2)	Positive

3.4. Production of enzyme, partial purification, and effect of environmental factors

In order to conclude the approximate optimum conditions for extracellular protease production by the isolate under study, *B. cereus* BC2, and to partially characterize the produced enzyme, we tested the effect of some important variable environmental conditions on the production of enzyme by the isolate and then the enzyme was partially purified. In all cases, the total protein concentration plus the enzyme activity were measured quantitatively. The tested variables were different temperatures, pH values, and NaCl concentrations. The isolate *B. cereus* BC2 was grown in TSB (which showed the best growth and enzyme activity when the isolate grown on for 48 hrs under shaking at 140 rpm) at 30˚, 35˚, 40˚, 45˚, 50˚, 55˚ and 60˚ C to determine its optimum growth temperature and enzyme activity. The isolate was also grown at various pH levels from 6-11. The isolate was finally grown in TSB solution with 1%, 3%, 5% and 7% (w/v) Sodium Chloride. Table (4) show that the optimum production was at pH 8, incubation temperature 55°C, and NaCl concentration of 5 % (w/v) where the highest protein concentration and the highest enzyme activity were recorded in each case. In case of incubation at pH 8 the recorded protein concentration was 2.3 mg mL^{-1} and the enzyme activity was 100.00 UmL^{-1}. While, in case of incubation at 55°C the recorded protein concentration was 2.5 mg mL^{-1} and the enzyme activity was 110.00 UmL^{-1}. Finally, in case of incubation at 5 % (w/v) NaCl the recorded protein concentration was 2.1 mg mL^{-1} and the enzyme activity was 90.00 UmL^{-1}. The activity of the partially purified enzyme has increased to about 1.5 fold where it reached to about 160.00 UmL^{-1} with about 3.10 mg mL^{-1} protein concentration recorded.

3.5. Antibiotic resistance and plasmid profile of the isolate *Bacillus cereus*BC2

The antibiotic resistance test of the isolate was studied by disc diffusion method. The isolate was classified as resistant or sensitive by the absence/presence of inhibition zone of growth around the antibiotic discs. Table (5) show that, the isolate *Bacillus cereus* BC2, was resistant to the antibiotic Cephalexin, Cefaclore, Penicillin, Ampicillin, and Amoxycillin. On the other hand, this isolate was sensitive to the antibiotic Erythromycin, Lincomycin, Amikacin, Chloramphenicol, Kanamycin, Neomycin, Tetracycline, and Streptomycin. Finally, plasmid DNA profile extracted from the isolate *Bacillus cereus* BC2 under study and separated by gel electrophoresis (Figure 1) show the absence of any free plasmid molecules within the vegetative cells of this isolate. Obviously, the role of plasmid in β-Lactam resistance of this isolate is missing here as discussed briefly below.

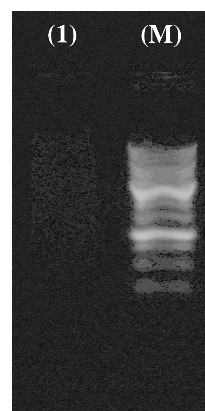

Figure 1. Plasmid profile of the isolate *Bacillus cereus* BC2; (M) is the DNA marker & (1) is the tested isolate sample.

Table 4. The effect of variable cultural conditions on protease production by *B. cereus* BC2.

Initial pH value	**Enzyme activity U mL^{-1}**	**Protein concentration mg mL^{-1}**
6	70.00	1.50
7	80.00	1.80
8	100.00	2.30
9	90.00	1.90
10	70.00	1.60
11	40.00	0.90
Temperature °C		
30	50.00	1.15
35	50.00	1.20
40	60.00	1.40
45	70.00	1.60
50	90.00	2.10
55	110.00	2.50
60	90.00	2.05
NaCl % (w/v)		
1	60.00	1.50
3	70.00	1.60
5	90.00	2.10
7	50.00	1.20

Table 5. The pattern of antibiotic resistance of the isolate *B. cereus* BC2.

Antibiotic concentration (*μg*/disc)	**Resistance pattern**
Cephalexin (30)	Resistant
Cefaclore (30)	Resistant
Penicillin (10)	Resistant
Ampicillin (10)	Resistant
Amoxycillin (10)	Resistant
Erythromycin (15)	Sensitive
Lincomycin (15)	Sensitive
Amikacin (30)	Sensitive
Chloramphenicol (30)	Sensitive
Kanamycin (30)	Sensitive
Neomycin (30)	Sensitive
Tetracycline (30)	Sensitive
Streptomycin (10)	Sensitive

4. Discussion

The *Bacillus cereus* group is composed of *B. anthracis, B. cereus, B. mycoides, B. pseudomycoides, B. thuringiensis* and *B. weihenstephanensis* (Nakamura, 1998 and Lechner *et al.,* 2006). Members of this group share many of their biochemical, morphological and very closely related gene sequence based on their 16S rRNA (Bavykin *et al*., 2004 and Wang *et al*., 2007). The genomic and proteomics analysis between members of this group yield important findings to understand their evolution and interrelationship. *B. thuringiensis* can be differentiated from *B. cereus* by the presence of the CRY crystal protein and plasmid encoded CRY genes thus loss of this gene would mean that *B. thuringiensis* could not be distinguished from *B. cereus* (Cherif *et al*., 2007).

In the current study, the isolated *Bacillus cereus* BC2 was identified, according to Sneath, (1986) and by using API system, and investigated as to produce protease activity, its antibiotic resistance pattern, and the possible existing free plasmid DNA molecules within the vegetative cells of this isolate. The initiative was to come closer to one from this very important and highly economic group of bacteria. This isolate was grown well on nutrient

agar plates (pH 8) after 24 hrs of incubation at 55°C. Also, this isolate was characterized by the presence of central ellipsoidal endospores (without parasporal crystals), aerobic growth, and high motility.

Extracellular protease activity of the isolate was measured qualitatively by caseinolytic zone diameter on skim milk agar plates (pH 8) at 55°C both *in vivo* and *in vitro* where it ranged between 20 - 25 mm. The optimum production conditions for extracellular protease by the isolate under study, *B. cereus* BC2 after 48 hrs under shaking at 140 rpm was at pH 8, incubation temperature 55°C, and NaCl concentration of 5 % (w/v) where the highest protein concentration and the highest enzyme activity were recorded in each case. The activity of the partially purified enzyme has increased to about 160.00 UmL^{-1} with about 3.10 mg mL^{-1} protein concentration recorded.

Similar studies were conducted to investigate the activity of Thermotolerant proteases such as those isolated by Suntornsuk *et al*., (2005) from a soil *Bacillus licheniformis* which had a high specific activity (218 U mg^{-}1), a molecular mass of 35 kDa, and an optimum pH and temperature of 8.5 and 60°C, respectively. This isolate hydrolyzed the soluble proteins casein and gelatin more efficiently than other substrates tested such as keratins. The results of Nadeem *et al*., (2007) indicated that enzyme produced by *B. lichniformis*-N2 is active within broad ranges of temperature and pH. These characteristics render its potential use in leather and detergent industries.

There are few studies of the sensitivity of *Bacillus* species to antibiotics even though they relate to the genus taxonomy. Ha *et al*., (2002) stated that *Bacillus* sp. KYJ 963 showed resistance to the antibiotics penicillin G producing an extracellular ß-amylase. A good and simple tool for differentiation of probiotic and wild-type *B. cereus* strains is their different resistance to Penicillin G and Tetracycline. The wild-type strains are resistant to Penicillin G and sensitive to Tetracycline. The probiotic strains behaved vice versa (Mietke *et al*., 2001).

In our study, the isolate *Bacillus cereus* BC2 was resistant to the *β*-Lactams; Cephalexin, Cefaclore, Penicillin, Ampicillin, and Amoxycillin. On the other hand, this isolate was sensitive to the antibiotic Erythromycin, Lincomycin, Amikacin, Chloramphenicol, Kanamycin, Neomycin, Tetracycline, and Streptomycin comprising the main protein synthesis inhibitors.

Even though Ombui *et al.,* (1996) did not prove the link between the presence of plasmids and the antimicrobial drug resistance of *B. cereus* strains isolated from milk, a tetracycline resistance gene on a potential mobile element, plasmid pBC16 (Palva *et al*., 1990), was demonstrated and sequenced. It was also demonstrated that resistance to antimicrobial substances methicillin, gentamicin, kanamycin and tetracycline, can be acquired by the transfer of the relevant plasmid between Gram-positive microorganisms, among which *B. cereus* falls (ECDC, 2002).

Generally, *Bacillus cereus* group genomes are usually 5.2–5.4Mb in length including a single circular chromosome and most strains carry one or several extrachromosomal plasmids that are responsible for the main phenotypic differences between the species. *Bacillus cereus* group organisms have been shown to carry a number of group I and group II introns, some of them exhibiting unusual properties (Tourasse *et al*., 2006). Tourasse and Kolstø (2008) stated that Group II introns are mainly disseminated via plasmids and can invade the host genome.

The different species comprising the *Bacillus cereus* sensu lato group are largely defined by differences in plasmid-encoded features, while the chromosomes have been shown to be similar in both gene content and gene order (Rasko *et al.,* 2005). The current species concept defines *Bacillus anthracis* as containing two plasmids, pXO1 and pXO2, that encode for the toxin and the capsule, respectively (Okinaka *et al*. 1999). *Bacillus thuringiensis* isolates contain plasmids that encode various isoforms of the Bt toxin (Schnepf *et al*., 1998). The plasmid profile of *Bacillus cereus* sensu stricto is extremely variable, and no well defined conserved members have been identified that could delineate the species. These plasmid-based species definitions have resulted in the classification of members of the *B. cereus* group that are not valid when molecular typing is applied and the suggestion that these three species should be regarded as a single species (Rasko *et al*., 2005).

Jensen *et al*., (2003) stated that *Bacillus cereus sensu lato* species group comprising *Bacillus anthracis*, *Bacillus thuringiensis* and *B. cereus* (*sensu stricto*), has previously been scrutinized regarding interspecies genetic correlation and pathogenic characteristics. So far, little attention has been paid to analyzing the biological and ecological properties of the three species in their natural environments. The study of Schlegelova *et al*., (2003) showed that almost all *B. cereus* isolates from food stuffs displayed low susceptibility to ampicillin, cephalothin, and to oxacillin and may be become vectors of resistance to antimicrobial agents via this pathogenic microorganism. Plasmids can be transferred horizontally from a plasmid-carrying cell to another cell that does not carry it through a process called conjugation. Some *Bacillus cereus*

strains contain a plasmid designated pBC16, which codes for tetracycline resistance. The protein that this plasmid codes for acts like a pump, forcing the tetracycline out of the cell before it can stop the bacterial protein synthesis (Nester *et al*., 2004).

In our study, plasmid DNA profile extracted from the isolate *Bacillus cereus* BC2 under study and separated by gel electrophoresis showed the absence of any free plasmid molecules within the vegetative cells of this isolate. Obviously, the role of plasmid in *β*-Lactam resistance of this isolate is missing here, so, we can say that the genes for *β*-lactamase is generally chromosomal located, although, some other unknown factors are still required for production regulation. However, there is still some doubt about the plasmid role since it was believed that the production of *β*-lactamases is generally extrachromosomal regulated as concluded from studies on antibiotic resistance of bacteria.

In earlier studies, the susceptibility of most *Bacillus anthracis* strains to β-lactam antibiotics was interesting considering that the closely related species *Bacillus cereus* and *Bacillus thuringiensis* typically produce β-lactamases and the *B. anthracis* genome harbors two β-lactamase genes, *bla1* and *bla2*. In addition to plasmid-associated traits, some species-specific phenotypes are due to differential expression of chromosomal genes. *B. cereus* and *B. thuringiensis* have certain phospolipase, hemolysin, protease, and β-lactamase activities that are generally not associated with *B. anthracis*. For example, penicillin susceptibility is a characteristic of *B. anthracis* that is commonly used to distinguish this species from *B. cereus* and *B. thuringiensis*, which typically exhibit inducible penicillin resistance. The genetic basis for β-lactam resistance in this group of organisms is unknown. Interestingly, all sequenced strains of *B. anthracis*, *B. cereus*, and *B. thuringiensis* contain β-lactamase genes (Bernhard *et al*., 1978).

5. Conclusion

In conclusion, the aim of the current study was to isolate and identify of one representative thermotolerant isolate from the *Bacillus cereus* group species. Also, in parallel, proteolytic activity and antibiotic resistance were investigated to evaluate the taxonomic potential of this isolate among the well studied representatives of this group including *Bacillus anthracis* and *Bacillus thuringiensis*. The idea that these three species are very closely related make them interrelated and the benefits of and/or harmful effects gained from can be regulated specially with the advent of plasmid handling and cloning. Further studies on such isolates will contain a stability study for some plasmid vectors which can be used as cloning vectors and also as shuttle vectors, for example, carrying a Bt toxin gene. Also, initial contamination levels by wild type strains and the toxigenic potential of *B. cereus* group bacteria, especially those commonly encountered in the vegetable industry, should be determined.

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6/7/2010